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RESEARCH ARTICLE

Serpin A1 and the modulation of type I collagen turnover: Effect of the C-terminal peptide 409–418 (SA1-III) in human dermal fibroblasts

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Abstract

The pharmacological modulation of collagen turnover is a strategy potentially useful in different skin conditions. The serine protease inhibitor Serpin A1 and portions of its C-terminal region have been investigated as collagen modulators. To clarify the mechanisms by which the C-terminal 409–418 peptide SA1-III increases extracellular type I collagen levels, to compare its activities range with that of the originator molecule Serpin A1, and to evaluate its efficacy in primary cultures from adult and aged human subjects. The different forms of type I collagen were analyzed by means of western blot in cell lysates and cell-conditioned media of primary human dermal fibroblasts obtained from subjects of different ages. Gelatin zymography was used to investigate the degrading enzymes. Cell viability and in vitro wound healing tests were used to evaluate cell proliferation. The SA1-III peptide increased extracellular collagen levels by reducing degradation, with no effect on cellular biosynthesis or cell proliferation mechanisms. A reduced level of MMP-2 and MMP-9 was also found in cell media upon peptide treatment. No peptide effect was detected on inflammatory mediators gene expression in resting and LPS-stimulated fibroblasts, or in the wound healing test. The SA1-III peptide is a good collagen modulator candidate, protecting collagen against degradation without detectable actions on biosynthesis, acting at reasonably low concentrations, and non-interfering with cell proliferation. It is effective in primary fibroblasts from young and aged subjects. These effects can prove useful in pathological and physiological skin conditions in which collagen degradation is excessive compared to the synthetic capacity.

Keywords: bioactive peptides; metalloproteases; serine proteases; skin aging; wound healing

Introduction

Interstitial type I collagen is the most diffuse collagen form in connective tissues. The pharmacological modulation of collagen turnover is a therapeutic strategy potentially useful in different pathological and physiological conditions, such as: genetically determined collagen deficiency states (type I osteogenesis imperfecta, Ehlers-Danlos syndrome) (Myllyharju and Kivirikko, 2001); excessive collagen deposition in fibrotic pathologies (Lopez et al., 2010; King et al., 2011); excessive collagen degradation in inflammatory conditions of bone and cartilage (Burrage et al., 2006). In the skin, excessive collagen type I degradation has been shown in chronic wounds (Caley et al., 2015); in skin photodamage and photoaging, ECM-digesting enzymes are activated and collagen degradation is increased (Pillai et al., 2005). In chronologically aged skin, dermal fibroblasts produce less ECM components, including type I collagen (Varani et al., 2006).

In this line, many efforts have been devoted to the characterization of the degradation pathways, that may vary according to the tissue and the pathological state. Matrix metalloproteases are known to play a pivotal role in extracellular collagen degradation: some of them, such as

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; FBS, fetal bovine serum; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloprotease; NHDF, neonatal human dermal fibroblasts; SDS, sodium dodecyl sulfate; TGF-β, transforming growth factor β

collagenases MMP-1, MMP-8, and MMP-13 are able to degrade fibrillar collagen acting on a single cleavage site and producing fragments of about 100 kDa (the so-called 3/4) and 30 kDa (1/4) (Williams and Olsen, 2009), whereas gelatinases like MMP-9 and MMP-2 cleave with higher efficiency soluble collagen, acting at the same site than classic collagenases (Aimes and Quigley, 1995; Bigg et al., 2007). However, MMP-2 and MMP-9 have been reported to be able to cleave the native triple-helix as well (Aimes and Quigley, 1995; Bigg et al., 2007). Tissue and plasma serine proteases are also involved in extracellular collagen degradation, both indirectly, by participating in the proteolytic cascade leading to MMP activation as in the case of plasminogen (Murphy et al., 1999) or trypsinogen-2 (Moilanen et al., 2003), and directly. Among these, trypsinogen-2 and neutrophil elastase have been shown to be able to degrade interstitial type I and III collagen acting on different sites from those of MMPs (Mainardi et al., 1980; Moilanen et al., 2003). A serine protease-dependent collagen degrading activity has been reported in joint synovial fibroblasts (Song et al., 2006).

Accordingly, serine protease inhibitors have attracted attention as possible collagen modulators. Among these, Serpin A1 (alpha1-antitrypsin, alpha1-proteinase inhibitor) is a major trypsin and elastase inhibitor, also endowed with potent anti-inflammatory activities: the coexistence of different biological activities in the same molecule can prove particularly useful in inflammation-associated collagen degradation, like that occurring in chronic wounds and radiation-damaged skin. Wound healing disturbances have indeed been reported in a subject deficient in alpha1-antitrypsin (Cathomas et al., 2015). Dabbagh et al. (2001) have shown increased procollagen production upon treatment of fibroblasts with Serpin A1. However, given the size of the molecule (a 52 kDa protein) and the associated delivery problems, many authors have investigated the biological activity of shorter fragments: the 36 aminoacid C-terminal portion, released by a proteolytic cleavage from the complex serpin-target protease, has been shown to have different biological effects independent of the antiprotease activity and rather mediated by modulation of gene expression: among these, a neutrophil chemotactic action that might be useful in wound healing (Joslin et al., 1992). The shorter A1-C26 portion has been shown to increase collagen extracellular levels upon treatment of fibroblasts in vitro (Congote et al., 2008). This region comprises aminoacids 393–418, and because it is further downstream of the SerpinA1 reactive center, its activity is not expected to be due to serine protease inhibition. However, putative cleavage sites for elastase and metalloproteases are present in the A1-C44 region (residues 409–410 and 412–413), that are included in the A1-C26 peptide sequence and might be involved in competitive inhibition of these collagen-degrading enzymes (Niemann et al., 1997). We have further dissected the C26 terminal analyzing the biological activity of three different overlapping 10-residue peptides, and selected the 409–418 fragment (called SA1-III) as the best collagen turnover modulator candidate, based on the physicochemical features and the efficiency in increasing the levels of extracellular type I collagen in cultures of neonatal human fibroblasts (Pascarella et al., 2016).

The first goal of the present work has been to shed light on the mechanisms of action underlying the effects of the SA1-III peptide. To this aim, we evaluated by means of western blot the different forms of collagen present in both cell lysates and cell-conditioned media. Compared to the ELISA method used in the previous work, this procedure allowed us to follow the fate of collagen in fibroblasts cultures from the newly synthesized pre-pro-procollagen to the degradation fragments originating extracellularly. Furthermore, by comparing the treatment effect on cell lysates with that on conditioned media, we were able to analyze separately the intracellular collagen pool, prevalently subjected to synthesis-associated changes, from the extracellular, modifiable by both synthesis changes and protease degradation. The proteolytic activities in the conditioned fibroblast media were also investigated by means of zymography.

The second goal was to evaluate whether other biological effects of the originator Serpin A1 besides collagen modulation, such as the anti-inflammatory and proliferative action, were maintained in the shorter C terminal sequence 409–418.

Finally, this work aimed to verify whether the effects previously shown in cultured human neonatal fibroblasts with the SA1-III peptide were still present in fibroblasts obtained from adult subjects of different ages, thus providing support for a possible use in the general population.

Methods

Peptide synthesis

SA1-III (sequence Ac-Met-Gly-Lys-Val-Val-Asn-Pro-Thr-Gln-Lys-NH2) was prepared by solid-phase peptide synthesis (SPPS) as previously described (Pascarella et al., 2016). Briefly, synthesis was performed manually on Rink-amide AM resin using the 9-fluorenyl-methoxycarbonyl/tert-butyl (Fmoc/tBu) strategy. After coupling the final amino acid to the resin, the peptide was N-terminally acetylated with acetic anhydride and then cleaved from the solid support with a mixture of TFA:H2O: TIS:EDT (94:2.5:1:2.5). The crude peptide was purified by semi-preparative RP-HPLC in order to obtain a chromatographic purity of 98% and lyophilized. Finally, SA1-III was characterized by analytical HPLC and electrospray ionization mass spectrometry (ESI-MS).

Cell cultures

Neonatal human dermal fibroblasts (NHDFs) were obtained from Lonza. Adult dermal fibroblast cultures were prepared from skin biopsies obtained from healthy donors of different ages, as described (Pani et al., 2009): C6 (male 36 years old), C3 (female 57 years old), C12 (female 84 years old). Informed written consent to use dermal cells for analysis was obtained from the subjects, according to the guidelines indicated by the local Ethical Committee and to the Declaration of Helsinki of 1975, as revised in 1983. All the cultures had undergone a similar number of passages (6–9) at the beginning of the experiments, and were maintained in Dulbecco's modified Eagle's medium (DMEM, Lonza) supplemented with 10% FBS (Gibco), 100 U/mL of penicillin G, 0.05 mg/mL streptomycin, and 2 mM glutamine (PAN-Biotech GmbH), at 37°C in a humidified incubator containing 5% CO_2.

Cells were seeded into 12-well plates (30,000 cells per well) and treated the following day with SA1-III (20 μM), TGF-β1 (10 ng/mL, positive control), or DMEM only (negative control). All treatments were conducted in the absence of FBS to avoid interference in electrophoretic migration. After 72 h incubation, culture media were collected, centrifuged at 250*g* for 5 min and the supernatants aliquoted and stored at −20°C for collagen analysis and zymography; cellular proteins were extracted in RIPA buffer containing 1% protease and phosphatase inhibitor cocktail (Sigma–Aldrich Chemicals) with the aid of a cell scraper. The lysates were then sonicated, clarified by centrifugation and supernatants collected and stored at −20°C. Protein content in the lysates was measured by using the Bio-Rad DC protein assay kit (Bio-Rad). In a subset of experiments with NHDFs, cellular proteins were recovered after trypsinization with Trypsin-EDTA (EuroClone), to digest ECM molecules attached to the cell surface. The obtained cell pellet was then dissolved in RIPA buffer as above to extract proteins.

Separate experiments were also conducted for total RNA extraction, treating attached cells with TRIzol reagent (Invitrogen). To induce an inflammatory state, cells were treated with LPS (10 μg/mL) for 24 h in DMEM with no FBS.

MTS viability assay

Cell viability assays were performed with the MTS test in 96-MW (8,000 cells/well) after 72 h treatments, according to the manufacturer's instructions (Promega).

Western blot analysis

Thirty-forty micrograms of lysate proteins for each sample were subjected to 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis separation (Bis-Tris Plus BOLT, Invitrogen) under standard dissociating and reducing conditions and transferred to polyvinylidene fluoride membranes (PVDF, Millipore). Collagen I and GAPDH proteins (the latter used as loading control) were determined by immunostaining with primary rabbit polyclonal antibodies: anti-type I collagen, ab34710, Abcam; anti-GAPDH, 14C10, Cell Signaling Technology) and suitable peroxidase-conjugated secondary antibodies (Sigma–Aldrich Chemicals). Protein bands were visualized using the enhanced chemiluminescence procedure with Immobilon Horseradish Peroxidase Substrate (Millipore) and immune-reactive bands were quantified by densitometric analysis using the Quantity-One software (Bio-Rad). Each density measure was normalized by using the corresponding GAPDH level as an internal control.

For the measurement of soluble collagen type I in culture media, 500 μL of each sample were concentrated 10 times by means of centrifugal filters with a 3 or 30 K cut-off (Amicon Ultra-0.5 mL, Millipore). About 20 μL of each sample were then used for western blot analysis. In the case of media, each density measure was normalized with the protein content of the cells in the corresponding well. As a reference control, human type I collagene (BD Biosciences) was run in some experiments along with the experimental samples. The employed molecular weight markers were Magic Mark (Invitrogen, visible in chemilunescence) and Page Ruler (Thermo Scientific, in visible light).

Gelatin zymography

Aliquots (10–20 μL) of culture media were mixed with Tris–Glycine SDS Native Sample Buffer (Invitrogen), electrophoresed through 10% Novex Zymogram Gelatin Gels (Invitrogen) and developed according to the manufacturer's instructions. The bands containing gelatinolytic activity of MMP2 and MMP9 appeared transparent in the otherwise blue gel. Bands were quantified using ImageJ software.

Real time PCR and wound healing test

See Supplementary Material (Supplementary Table S1).

Statistical analysis

The values are expressed as the mean ± standard error. Statistical analyses were performed using one-way ANOVA along with Bonferroni's *post hoc* test. The Student's *t* test has been used for the analysis of paired data. $P \leq 0.05$ was considered to be a statistically significant difference.

Results

Cell culture morphology

In standard culture conditions, all the adult cultures were able to reach confluence, although in a longer time

compared to NHDFs. Cell proliferation curves are shown in Supplementary Figure S1. No apparent detrimental effect of the 72 h serum-free incubation was observed (Supplementary Figure S1 and Supplementary Table S2). The culture from the younger adult, C6, exhibited a spindle-shaped morphology similar to that of NHDFs; C3 cultures showed some enlarged cells; C12 cultures showed a more flattened and enlarged morphology (Supplementary Figure S1). The 72 h treatments with the peptide or TGF-β did not induce detectable morphological changes, although with the latter the expression of α-smooth actin was increased as expected (data not shown).

Collagen detection by western blot

In Figure 1, a comparison between the collagen bands found in cell-conditioned media and purified type I human collagen is shown. It can be noticed that the reference collagen shows two close bands in the vicinity of the 100 kDa molecular weight, as expected for alpha 1 and 2 chains (predicted weight 100 and 98 kDa respectively, Uniprot data, http://www.uniprot.org/). Heavier forms are also visible in the 200–300 kDa range, reasonably representing dimers or trimers, and a lower band in the 60 kDa range, attributable to degradation. In the cell-conditioned media a different pattern was observed, with two close bands in the 140 kDa range representing the most abundant forms, and three main lower molecular weight fragments around 100, 70, and 60 kDa.

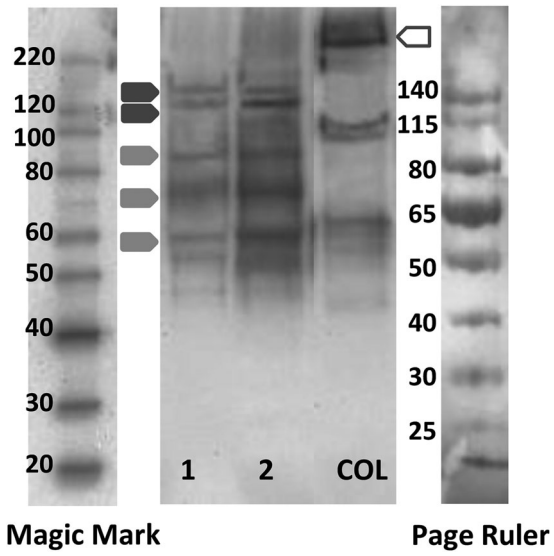

Figure 1 Membrane probed with the employed Abcam anti-collagen type I antibody. Lane 1 and 2: 10-times concentrated samples of NHDF-conditioned media; lane 3 (COL): standard human type I collagen. Molecular weights (kDa) defined by using Magic Mark in chemiluminescence and Page Ruler in visible light are shown. Dark arrows indicate the collagen bands used for quantification of collagen (>140 kDa), and light gray arrows indicate fragments (molecular weights around 100, 70, and 60 kDa) in the cell conditioned media. The white arrow on the right indicates heavier collagen forms in the reference standard. A 60 kDa degradation fragment was also detected in the standard COL.

The heavier bands molecular weights are compatible with the unprocessed or partially processed procollagen forms (139 and 129 kDa for pro-alpha1 and 2 respectively, Uniprot data).

Effect of SA1-III treatment on collagen in cell lysates

The same pattern was observed in cell lysates, with the fragments being less evident (Figure 2). In this regard, a difference was observed between NHDFs and adult fibroblasts, in the recovery of collagen with the standard method for cellular protein collection (i.e., direct cell solubilization with RIPA buffer): in adult fibroblasts the prevalent collagen forms detected were the two heavier bands (membrane examples in Figure 2, right panels), expected to be the main intracellular forms; instead, proteins prepared from NHDFs contained not only these newly synthesized forms, but also smaller fragments that are supposed to be originated extracellularly (membrane examples in Figure 2, left panel). To analyze the truly intracellular forms, we then decided to perform a group of experiments in which the cells were pre-treated with trypsin: cells were thus detached and recovered by centrifugation as in routine cell passage, and then the cell pellet was solubilized in RIPA buffer to obtain cellular proteins. Under these conditions, the lighter collagen forms detected upon western blot resulted to be drastically reduced in NHDF cellular proteins (membrane example in Figure 2, left panel).

The quantification of the procollagen bands in both adult fibroblasts and pre-trypsinized NHDFs showed no difference between control and peptide treatment, indicating no effect of SA1III on collagen intracellular levels. Instead, the positive control TGF-β induced a consistent and significant increase of both the procollagen forms in all the cultures, as expected.

Effect of SA1-III treatment on collagen in cell-conditioned media

In the culture media, the treatment with the peptide increased both the heavier forms compared to controls in all the cultures (Figure 3). NHDFs resulted to produce higher levels of collagen in the medium both in control conditions and upon stimulation by the peptide, compared to the adult cultures. The stimulating effect of the positive control TGF-β was rather homogeneous across all cultures. We also noticed that the degradation bands clearly detectable in NHDF media, were only faintly visible in the media conditioned by adult fibroblasts. The quantification of the most evident fragments, around 100, 75, and 60 kDa, in NHDF-conditioned media showed a strong decrease of all

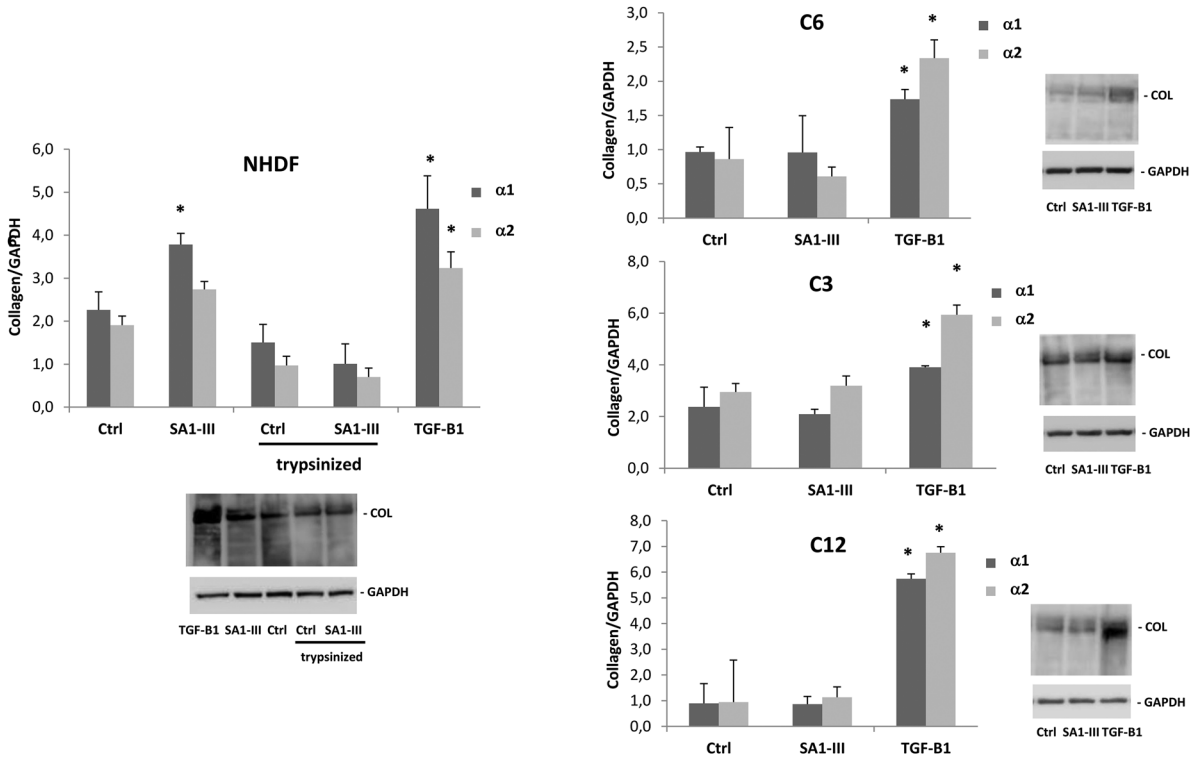

Figure 2 Effect of SA1-III (20 μM) peptide and TGF-beta (10 ng/mL) on collagen production in fibroblasts (72 h treatment). Collagen amount was quantified by western blot in cell lysates using the two bands (>120 kDa) corresponding to unprocessed α1 and α2 chains, and GAPDH protein expression as the internal loading control. The results are shown for NHDFs (left panel) and three adult fibroblast cultures (C6, C3, and C12, right panels). Data are the mean ± SE of 3–4 experiments. Trypsinized NHDF indicate cells that have been treated with trypsin before protein collection (see Methods section). An example of blotted membranes is shown for each graph. $^*P < 0.05$ statistically significant difference from the corresponding control (Ctrl).

this forms upon SA1-III treatment, whereas TGF-β tended to increase the amount of these fragments in the medium.

Effect of SA1-III treatment on MMP activity in cell-conditioned media

In view of a possible effect of the peptide on extracellular collagen degradation, we investigated protease activity in concentrated media by means of zymography: no activity was found in casein gels, indicating that at least with this method serine protease activity in fibroblast media was undetectable. In gelatin gels, MMP-9 and MMP-2 activities were detected, that resulted to be reduced in media conditioned by cells treated with the peptide (Figure 4), and were not modified by treatment with TGF-β.

Effect of SA1-III treatment on the expression of inflammatory mediators

The potential anti-inflammatory activity of the peptide was investigated both at rest and upon LPS stimulation, by means of PCR evaluation of IL-1β, IL-6, and TNF-α gene expression, in NHDF and C6 cultures. LPS did induce an increase in the expression of all these inflammatory mediators, which resulted to be stronger in NHDFs than in C6 cells. However, the peptide did not modify their expression either in control or in stimulated conditions (Supplementary Figure S2).

Effect of SA1-III treatment on basal and stimulated cell proliferation and migration

The potential of the peptide to induce cell proliferation and wound healing was evaluated in NHDFs with the MTS test for cell viability and with the in vitro wound test. No difference between peptide treatment and control was found in the MTS test either in the presence or absence of serum (Supplementary Table S2), indicating both absence of toxicity and lack of proliferation induction. Similarly, no effect of the peptide was found on stimulated proliferation and migration in the wound healing test (Supplementary Figure S3).

Discussion

We have studied by means of western blot the modulation of intracellular and extracellular type I collagen in primary

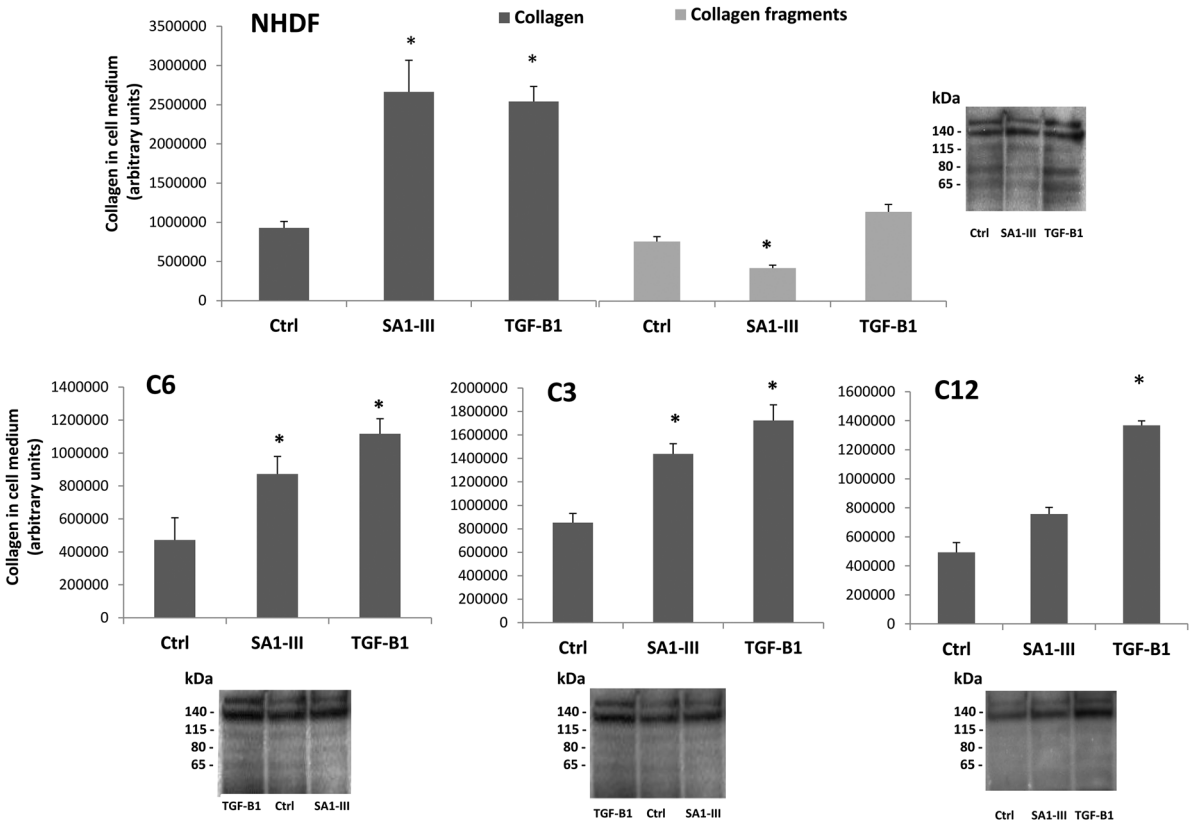

Figure 3 Effect of SA1-III (20 μM) peptide and TGF-beta (10 ng/mL) on soluble collagen levels in cell-conditioned medium (72 h treatment). The results are shown for NHDFs (upper panel) and for three adult fibroblast cultures (C6, C3, and C12, lower panels). Collagen amount was quantified by western blot in 10-times concentrated samples of conditioned media using the two bands (>120 kDa) corresponding to unprocessed α1 and α2 chains (darker gray columns) and data are shown as the sum of the intensity of the two bands; the three main fragments (<100 kDa) were quantified in NHDFs (lighter gray columns): data are shown as the sum of the intensity of the three bands. Data are the mean ± SE of 3–4 experiments. An example of blotted membrane is shown in panel a. $^*P < 0.05$ statistically significant difference from the corresponding control (Ctrl).

cultured human dermal fibroblasts obtained from subjects of very different ages, from neonatal to 84 years, upon treatment with the Serpin A1 C-terminal peptide 409–418, named SA1-III, previously described by us as a collagen modulator candidate (Pascarella et al., 2016). We have shown previously a dose-response effect of the peptide on collagen levels in fibroblast-conditioned medium, with an EC50 of 10 μM. The concentration used, 20 μM, was selected in order to obtain a substantial effect, making the definition of the involved mechanisms easier. In any case, this is an attainable local concentration upon topical dermal administration, since the peptide will be formulated using suitable penetration enhancers, which will improve skin adsorption without interfering with bioactivity (Badenhorst et al., 2014). The observed effects were quite consistent across the cultures, although neonatal fibroblasts (NHDF) tended to proliferate more rapidly and to produce higher levels of total collagen in the extracellular medium.

The western blot pattern of collagen forms within and outside the cells has not been investigated in much detail. In one work with cat cardiac fibroblasts, Poobalarahi et al. (2006) showed a pattern similar to the one described in the present work: beside the heavier bands interpreted by these authors as pre-pro and pro-collagen, they also detected the final collagen chains, which were not clearly visible in our blots. This difference is possibly due to the different cell type, experimental conditions and antibody used.

The present experiments showed no difference between treatment and control in the two prevalent intracellular forms, presumably corresponding to unprocessed or partially processed procollagen molecules (Prockop and Kivirikko, 1995). On the contrary, TGF-β, known to stimulate collagen synthesis, induced an increase of both forms, as expected. These data led us to exclude that the peptide might have prominent effects on collagen synthesis. It is of notice that NHDFs behaved differently from the adult-derived cultures, in that they appeared to rapidly produce a net of extracellular matrix trapping secreted and degraded collagen forms on the cell surface. For this reason, for these cells it was necessary to use trypsin to break up the

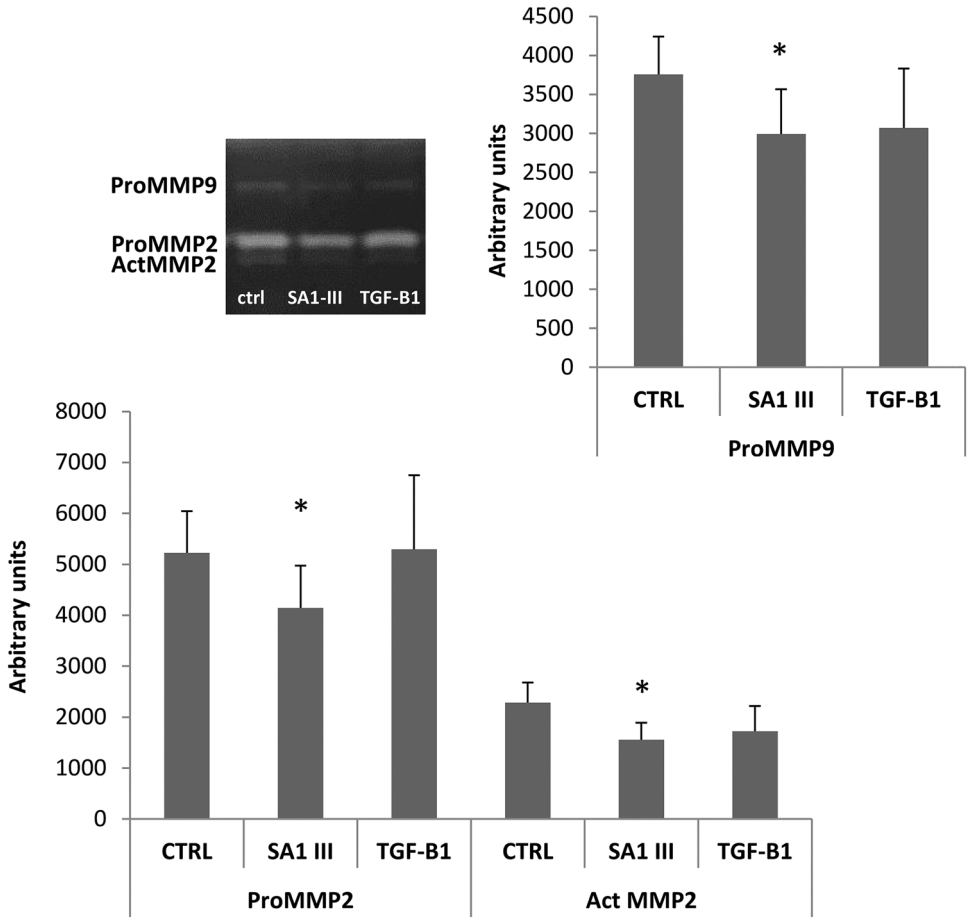

Figure 4 Effect of SA1-III (20 μM) peptide and TGF-beta (10 ng/mL) on the secretion and activation of gelatinases MMP-9 (upper right panel) and MMP-2 (lower panel) in culture medium. Enzyme activity was quantified by gelatin zymography in 10-times concentrated samples of 72 h-conditioned media. The results have been pooled for the three adult fibroblast cultures (C6, C3, and C12) and NHDFs. Data are the mean ± SE of eight experiments (two for each culture). An example of stained gel prepared from NHDF-conditioned media is shown in the upper left panel. $^*P < 0.05$ statistically significant difference (Student's *t* test for paired data) from the corresponding control (Ctrl).

surface-bound collagen and analyze the truly intracellular forms.

The method that we employed allows the measurement of soluble collagen forms, whereas the fibrillar collagen that the cells produce in the culture is left behind during medium recovery and protein preparation, and remains undetected. Thus, in the cell-conditioned medium we have been able to detect bands corresponding to newly released procollagen molecules, the two prevalent forms, and fragments originating from proteolytic cleavage of both procollagen and collagen. In particular, we found a series of partially degraded forms, among which the most evident corresponded to molecular weights of 100, 75, and 60 kDa respectively. Considering that the so-called 3/4 fragment originates from a cut between position 953 and 954 of procollagen (Williams and Olsen, 2009), according to UniProt data (http://www.uniprot.org/), this might be represented by the fragment we detected around 100 kDa. The same cut brought about on final fibrillar collagen could produce the lower weight fragments.

The peptide increased the level of the intact procollagen forms to a similar extent than TGF-β· However, at variance with TGF-β, it decreased all the three measured fragments compared to control levels, indicating reduced cleavage by extracellular MMPs and possibly by other collagen-degrading enzymes. This result prompted us to investigate the activity of the proteases present in fibroblast-conditioned media.

MMP-2 and MMP-9 were consistently detected in all cultures by zymography performed on extracellular medium samples, in agreement with the presence of the 3/4 fragment in the medium. The zymographic analysis of samples conditioned by cells treated with SA1-III showed a reduced gelatinolytic signal compared to samples conditioned by control cells, indicating reduced secretion of both the gelatinases by peptide-treated fibroblasts. The possibility that the peptide might additionally exert a direct competitive

inhibition on MMPs as previously hypothesized based on the work of Niemann et al. (1997) cannot be excluded.

Furthermore, other proteases contributing to collagen degradation might be present in fibroblast conditioned media under these experimental conditions, that might have remained undetected due to sensitivity limits of the employed methods: for example we have not detected any signal in casein zymography, a method known to be less sensitive than that performed with gelatin. Thus, a contribution of serine proteases to the observed extracellular collagen degradation pattern and to the peptide effects cannot be ruled out.

The data described so far indicate that the peptide SA1-III maintains the ability to increase collagen production previously described for the originator protein serpin A1 (Dabbagh et al., 2001; Fumakia and Ho, 2016) and for higher molecular weight fragments including SA1-III sequence (Congote et al., 2008). Other activities such as stimulation of proliferation and in vitro wound healing (Fumakia and Ho, 2016) do not appear to be conserved within the 409–418 sequence. As for the anti-inflammatory action, we have not found any effect of the peptide on IL-1β, IL-6, and TNF-α gene expression either at rest or in pro-inflammatory conditions (LPS stimulation). However, considering the prominent role played by MMPs in inflammatory conditions, the decreased secretion of MMP-2 and -9 brought about by the peptide can be regarded as a useful additional activity to be exploited in pathological conditions such as low-grade inflammation in aging and photodamaged skin (Pillai et al., 2005; Varani et al., 2006) and chronic wounds (Caley et al., 2015). For the latter, native collagen dressings have been put forward to improve tissue healing (Wiegand et al., 2016). Protecting endogenous collagen from excessive degradation might represent an alternative useful strategy.

The possibility of using the SA1-III peptide to treat different skin conditions is also supported by the fact that the described effects were present not only in neonatal but also in fibroblasts obtained by adult and aged subjects.

In conclusion, the present work confirms that the SA1-III peptide is a good collagen modulator candidate, active at reasonably low concentrations, and non-interfering with cell proliferation. Furthermore, we have shown its activity in adult fibroblasts, and demonstrated a protective effect against collagen degradation without detectable actions on the biosynthetic mechanisms. These effects can prove particularly useful in pathological and physiological conditions in which collagen degradation is excessive compared to the synthetic capacity of the cell.

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Conflict of interest

The authors declare no conflict of interest.

References

Aimes RT, Quigley JP (1995) Matrix metalloproteinase-2 is an interstitial collagenase. Inhibitor-free enzyme catalyzes the cleavage of collagen fibrils and soluble native type I collagen generating the specific 3/4- and 1/4-length fragments. J Biol Chem 270: 5872–6.

Badenhorst T, Svirskis D, Zimei W (2014) Pharmaceutical strategies for the topical dermal delivery of peptides/proteins for cosmetic and therapeutic applications. Austin J Pharmacol Ther 2: 1–0.

Bigg HF, Rowan AD, Barker MD, Cawston TE (2007) Activity of matrix metalloproteinase-9 against native collagen types I and III. FEBS J 274: 1246–55.

Burrage PS, Mix KS, Brinckerhoff CE (2006) Matrix metalloproteinases: role in arthritis. Front Biosci 11: 529–43.

Caley MP, Martins VL, O'Toole EA (2015) Metalloproteinases and wound healing. Adv Wound Care (New Rochelle) 4: 225–34.

Cathomas M, Schuller A, Candinas D, Inglin R (2015) Severe postoperative wound healing disturbance in a patient with alpha-1-antitrypsin deficiency: the impact of augmentation therapy. Int Wound J 12: 601–4.

Congote LF, Temmel N, Sadvakassova G, Dobocan MC (2008) Comparison of the effects of serpin A1, a recombinant serpin A1-IGF chimera and serpin A1 C-terminal peptide on wound healing. Peptides 29: 39–46.

Dabbagh K, Laurent GJ, Shock A, Leoni P, Papakrivopoulou J, Chambers RC (2001) Alpha-1-antitrypsin stimulates fibroblast proliferation and procollagen production and activates classical MAP kinase signalling pathways. J Cell Physiol 186: 73–81.

Fumakia M, Ho EA (2016) Nanoparticles encapsulated with LL37 and serpin A1 promotes wound healing and synergistically enhances antibacterial activity. Mol Pharm 13: 2318–31.

Joslin G, Griffin GL, August AM, Adams S, Fallon RJ, Senior RM, Perlmutter DH (1992) The serpin-enzyme complex (SEC) receptor mediates the neutrophil chemotactic effect of alpha-1 antitrypsin-elastase complexes and amyloid-beta peptide. J Clin Invest 90: 1150–4.

King TE, Jr., Pardo A, Selman M (2011) Idiopathic pulmonary fibrosis. Lancet 378: 1949–61.

Lopez B, Gonzalez A, Diez J (2010) Circulating biomarkers of collagen metabolism in cardiac diseases. Circulation 121: 1645–54.

Mainardi CL, Hasty DL, Seyer JM, Kang AH (1980) Specific cleavage of human type III collagen by human polymorphonuclear leukocyte elastase. J Biol Chem 255: 12006–10.

Moilanen M, Sorsa T, Stenman M, Nyberg P, Lindy O, Vesterinen J, Paju A, Konttinen YT, Stenman UH, Salo T (2003)

Tumor-associated trypsinogen-2 (trypsinogen-2) activates procollagenases (MMP-1, -8, -13) and stromelysin-1 (MMP-3) and degrades type I collagen. Biochemistry 42: 5414–20.

Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, Gavrilovic J (1999) Mechanisms for pro matrix metalloproteinase activation. APMIS 107: 38–44.

Myllyharju J, Kivirikko KI (2001) Collagens and collagen-related diseases. Ann Med 33: 7–21.

Niemann MA, Baggott JE, Miller EJ (1997) Binding of SPAAT, the 44-residue C-terminal peptide of alpha 1-antitrypsin, to proteins of the extracellular matrix. J Cell Biochem 66: 346–57.

Pani A, Dessi S, Diaz G, La Colla P, Abete C, Mulas C, Angius F, Cannas MD, Orru CD, Cocco PL, Mandas A, Putzu P, Laurenzana A, Cellai C, Costanza AM, Bavazzano A, Mocali A, Paoletti F (2009) Altered cholesterol ester cycle in skin fibroblasts from patients with Alzheimer's disease. J Alzheimers Dis 18: 829–41.

Pascarella S, Tiberi C, Sabatino G, Nuti F, Papini AM, Giovannelli L, Rovero P (2016) Serpin A1 C-terminal peptides as collagen turnover modulators. ChemMedChem 11: 1850–5.

Pillai S, Oresajo C, Hayward J (2005) Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation—a review. Int J Cosmet Sci 27: 17–34.

Poobalarahi F, Baicu CF, Bradshaw AD (2006) Cardiac myofibroblasts differentiated in 3D culture exhibit distinct changes in collagen I production, processing, and matrix deposition. Am J Physiol Heart Circ Physiol 291: H2924–32.

Prockop DJ, Kivirikko KI (1995) Collagens: molecular biology, diseases, and potentials for therapy. Annu Rev Biochem 64: 403–34.

Song F, Bergdoll AS, Windsor LJ (2006) Temporomandibular joint synovial fibroblasts mediate serine proteinase dependent Type I collagen degradation. Biochim Biophys Acta 1760: 1521–8.

Varani J, Dame MK, Rittie L, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ (2006) Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. Am J Pathol 168: 1861–8.

Wiegand C, Buhren BA, Bunemann E, Schrumpf H, Homey B, Frykberg RG, Lurie F, Gerber PA (2016) A novel native collagen dressing with advantageous properties to promote physiological wound healing. J Wound Care 25: 713–20.

Williams KE, Olsen DR (2009) Matrix metalloproteinase-1 cleavage site recognition and binding in full-length human type III collagen. Matrix Biol 28: 373–9.

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Supporting Information

dditional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Upper panel: proliferation curves of the four cultures in standard conditions (10% FBS). The Population Doubling (PD) increment was calculated as described below (Methods). Data are expressed as mean ± SE of three experiments. Lower panel: photomicrographs of control cell cultures after 72 h incubation in FBS-free medium: examples of neonatal (NHDF) and adult fibroblasts (C6, C3, and C12). NHDF, C6, and C3 cultures show a spindle-like shape typical of dermal fibroblasts. In C3 cultures, some enlarged cells were observed (arrows). In C12 cultures, the spindle shape is much less evident, and cells appear flattened and enlarged. Scale bar: 80 μ.

Figure S2. Effect of SA1-III (20 μM) peptide on the LPS-induced expression (mRNA level) of IL-1β, IL-6, and TNFα in NHDFs (left panels) and C6 fibroblasts (right panels). Cells were treated with LPS (10 μg/mL) for 24 h in the presence or absence of the peptide. Data are the mean ± SE of two experiments for each culture. No statistically significant difference was found between SA1-III treatment and the respective controls.

Figure S3. Effect of SA1-III (20 μM) peptide in the wound healing test. NHDF fibroblasts were wounded at time 0 (upper microphotographs) and let recover for 24 h (lower microphotographs) in control condition (left) and upon SA1-III peptide treatment (right). The quantification made with Image J program is shown in the graph on the right: wound closure is expressed as percentage of the initial wound (mean ± SE of three experiments).

Table S1. Primer sequences used for Real Time PCR experiments.

Table S2. Cell viability tested with MTS.